

Potassium Nitrite-Toxicity & Teratogenicity Studies in Avian Embryos-FDA Contract
#71-330 No Date

D23

POTASSIUM NITRITE

TOXICITY and TERATOGENICITY STUDIES
in AVIAN EMBRYOS

FDA CONTRACT #71-330

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STUDIES on the TOXICITY and TERATOGENICITY of POTASSIUM NITRITE

SUMMARY and CONCLUSIONS

Potassium nitrite (71-10) was toxic to avian embryos in each of the four test protocols employed in these studies. LD-50 estimates of 278.9, 47.1 and 387.4 mg/kg were obtained from the probit analyses program for the air cell-0 hrs, air cell-96 hrs and yolk-96 hrs series, respectively.

The data on the occurrence of abnormal embryos resulting from potassium nitrite injections failed to indicate a statistically significant increase as a result of any of the individual dose levels employed up to 1000 mg/kg. In the yolk-96 hr series, a comparison of all dose levels with water injected controls produced a significant chi-square value ($P < 0.05$). Potassium nitrite in this series produced an incidence of 1.73% abnormal embryos in comparison with 0.47% obtained with water injection. A comparison of head, skeletal, visceral and limb abnormalities in this same series (yolk-96 hrs) failed to produce a statistically significant chi-square value ($P > 0.05$).

GENERAL PROCEDURES

The protocols as specified under FDA Contract #71-330 were followed in the investigation of toxicity and potential teratogenicity of the specified substance. The toxicity of the substance was evaluated from the percentage hatch of embryos injected either in the air cell or yolk at either zero hours (^{RE}post-incubation) or after 96 hours incubation to provide four separate evaluations.

EGG SOURCE AND HANDLING

All eggs used in these investigations were from Shaver Starcross pullets housed at the Poultry Research Center of the University of Arizona in Tucson. The parent stock was maintained on the University of Arizona breeder diet which had been formulated to provide more than adequate amounts of all the known nutrients required by the breeding hen.

The feed was specially prepared to assure no contaminations and did not contain any additive drugs such as antibiotics. All eggs prior to use (within 48 hours of lay) were candled to remove any containing blood spots, abnormal air cells or abnormal shells, and only clean eggs ranging in weight from 23 - 26 ounces per dozen were used.

The supply flock was tested to assure the absence of Pullorum and Mycoplasma gallisepticum.

The eggs were incubated in forced draft Jamesway 252 machines with automatic temperature and humidity controls and an automatic turning device.

COMPOUND HANDLING FOR INJECTION

The substance tested was solubilized in a number of the prescribed solvents in order to determine the maximum concentrations which could be employed. Where possible, water was the solvent of choice. Maximum

injection volume was 0.05 ml. and all solvents and glassware were autoclaved prior to preparation of the solutions for use. The dose levels were administered with a microliter syringe using sterilized needles.

The preliminary range-finding studies using each of the administration routes and times were carried out with 10 - 25 eggs per dose level and included solvent controls, untreated controls and either drilled or pierced controls.

The actual dose-response protocol was carried out in two or more injections on different days to produce a minimum of 100 eggs at each dose level in five or more levels selected from the range- finding studies.

EXAMINATIONS OF EMBRYOS AND CHICKS

Eggs were candled daily and the dead embryos removed, examined and any abnormalities recorded. Five chicks from each dose level in each hatch were X-rayed to determine any skeletal abnormalities. Additional eggs injected at the approximate LD-50 level and an additional level below that were incubated and embryos at 8, 14, 17 days and hatch chicks removed for histopathological examinations.

In additional studies representative chicks from the dose-response protocol were saved. These chicks were housed in electrically-heated battery brooders with raised wire floors and fed University of Arizona diets. Feed consumption and growth rates were evaluated at 6 weeks of age and a sample of the birds sacrificed for gross and histopathological examinations.

The remaining birds in each group were maintained to 6 months of age and then sacrificed.

DATA HANDLING

All data were coded on forms provided by FDA for computer input. In addition to summaries of mortalities and abnormalities, a number of statistical evaluations were carried out. These statistical analyses included the following for both mortality and the incidence of abnormal embryos:

1. Chi-square tests for all dose levels and for each level against the solvent control.
2. Linear regression analyses + chi square test of linearity.
 - a. % response against dose
 - b. % response against log dose
 - c. log % response against dose
 - d. arcsin transformation against dose
 - e. arcsin transformation against log dose
3. Log dose against Probit using Finney's maximum likelihood method.
 - a. Where significant, the LD-30, 50, 70 and 90's were estimated with 95% confidence intervals.
4. One-way analyses of variance.
5. Linear regression with replication.

Potassium Nitrite (71-10) was solublized in deionized water for use in the test protocols; a maximum dose level of 1000 mg/kg was employed.

MORTALITY

Mortality data obtained in the four test procedures are shown in Tables 1 - 4. Chi-square analyses of these data indicated that dose levels of 300 mg/kg and above administered in the air cell prior to incubation produced significant ($P \leq 0.05$) increases in embryo mortality. Air cell-96 hour administration at levels of 20 mg/kg and above were toxic (Table 5). In the two test protocols involving yolk injections, 0 hr administration of potassium nitrite significantly increased mortality at levels of 200 mg/kg and higher, while 300 mg/kg were required to produce a significant increase in mortality when injected into the yolk at 96 hours.

Linear regression analyses of log dose against probit of mortality produced LD-50 estimates of 278.9, 47.1 and 387.4 mg/kg, respectively, for air cell-0 hrs, air cell-96 hrs and yolk-96 hrs (Table 6). The data obtained in the yolk-0 hr series did not yield a significant linear regression. These data indicate that potassium nitrite was toxic to avian embryos under the conditions of these studies.

TERATOLOGY

The occurrence of abnormal embryos and those showing head, skeletal, visceral and limb abnormalities are shown in Tables 1 - 4. Chi-square analyses of these data (Table 7) failed to indicate that any of the individual dose levels up to 1000 mg/kg produced a significant increase in the occurrence of abnormal embryos. Chi-square tests of all dose levels in comparison with the solvent injected control groups resulted in significant values ($P \leq 0.05$) for both the air cell and yolk 96 hour injection time (Table 7). In the air cell-96 hour series, those embryos injected with potassium nitrite showed

significantly less abnormalities than was obtained with the water injected control embryos. A total of 1.11% of the embryos receiving potassium nitrite were abnormal while 2.18% of those injected with water were classified as abnormal (Table 2). Injections of potassium nitrite into the yolk at 96 hours did significantly increase the incidence of abnormal embryos with a total of 1.73% showing abnormalities, while only 0.47% of those injected with water were abnormal (Tables 4 and 7).

Probit analyses of the data obtained for abnormal embryo incidences failed to show a statistically significant linear regression against log dose (Table 8).

Chi-square comparisons of the occurrence of H-S-V-L abnormalities failed to indicate a significant increase for any of the dose levels employed up to 1000 mg/kg (Table 9). Chi-square analyses of all dose levels in comparison with solvent controls yielded statistically significant ($P < 0.05$) chi-square values for the air cell-96 hr series. The air cell-96 hour series showed significantly ($P < 0.05$) less H-S-V-L abnormalities in those embryos injected with potassium nitrite with 0.89% in comparison with 2.18% H-S-V-L abnormalities in the water injected controls.

The individual teratogenic findings for the four test protocols are shown in Table 10.

The teratological data obtained in the air cell-96 hour series suggests that potassium nitrite may be teratogenic in the avian embryo.

POST HATCH DATA

Among those chicks injected with potassium nitrite and maintained to six months of age, no significant differences were obtained in weight at hatching or after either six weeks or six months in comparison with either the water injected controls or untreated groups. Feed consumption was normal and sexual maturity occurred at approximately the same time in all groups (Table 11).

TABLE I
K Nitrite

in Water

Air Cell - 0 hrs

Dose, ppm	No. Fertile	Mortality % #		Abnormal		Abnormalities by category						
						Head % #	Skeletal % #	Viscera % #	Limbs % #	Struc- tural % #	Toxic Response % #	Functional % #
				Total % #	H-S-V-L % #							
1000. 0.0	109	99.08	108	0.00 0	0.00 0							
500. 0.0	116	98.27	114	1.72 2	0.86 1	0.86 1				0.86 1		
300. 0.0	116	68.96	80	1.72 2	1.72 2	0.86 1		0.86 1				
200. 0.0	152	34.21	52	3.28 5	0.00 0							
100. 0.0	117	17.94	21	0.85 1	0.85 1	0.85 1						
0.0	143	24.47	35	0.00 0	0.00 0							
Drilled led	230	13.47	31	1.30 3	1.30 3	0.43 1	0.86 2					
Drilled eated	631	10.93	69	0.79 5	0.47 3	0.31 2		0.15 1		0.31 2		0.15 1

SUMMARY - ALL DOSE LEVELS

610	61.47	375	1.64 10	0.66 4	0.49 3		0.16 1		0.16 1		
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TABLE 2
K Nitrite

in Water

Air Cell - 96 hrs

se, pm	No. Fertile	Mortality % #		Abnormal Total % #		H-S-V-L % #		Abnormalities by category						
								Head % #	Skeletal % #	Viscera % #	Limbs % #	Struc- tural % #	Toxic Response % #	Functional % #
1000. 0.0	28	100.00	28	0.00	0	0.00	0							
500. 0.0	110	100.00	110	0.00	0	0.00	0							
300. 0.0	162	96.91	157	0.00	0	0.00	0							
200. 0.0	162	91.35	148	4.32	7	4.32	7	1.23	2		3.08	5	0.61	1
100. 0.0	163	64.41	105	0.00	0	0.00	0							
80. 0.0	48	95.83	46	2.08	1	0.00	0						2.08	1
60. 0.0	43	76.74	33	0.00	0	0.00	0							
20. 0.0	134	34.32	46	0.74	1	0.00	0						0.74	1
10. 0.0	52	3.84	2	1.92	1	1.92	1				1.92	1		
drilled 0.0	183	12.56	23	2.18	4	2.18	4	1.63	3		0.54	1	0.54	1
drilled ed 0.0	373	6.43	24	0.00	0	0.00	0							
untreated ated 0.0	631	10.93	69	0.79	5	0.47	3	0.31	2		0.15	1	0.31	2
														0.15 1

SUMMARY - ALL DOSE LEVELS

902	74.83	675	1.11	10	0.89	8	0.22	2			0.67	6		0.33	3
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TABLE 3
K Nitrite

in Water

Yolk - 0 hrs

Dose, ppm	No. Fertile	Mortality % #		Abnormal		Abnormalities by category						
						Head % #	Skeletal % #	Viscera % #	Limbs % #	Struc- tural % #	Toxic Response % #	Functional % #
				Total % #	H-S-V-L % #							
500	100	100.00	100	0.00 0	0.00 0							
300	119	72.26	86	3.36 4	3.36 4		0.84 1	0.84 1	1.68 2	0.84 1	0.84 1	
200	99	37.37	37	0.00 0	0.00 0							
199.8	20	45.00	9	0.00 0	0.00 0							
100	100	49.00	49	0.00 0	0.00 0							
99.6	20	60.00	12	0.00 0	0.00 0							
19.8	20	25.00	5	5.00 1	10.00 2	5.00 1		5.00 1				
10	100	39.00	39	1.00 1	0.00 0						1.00 1	
9.96	20	25.00	5	0.00 0	0.00 0							
0.0	224	53.57	120	0.89 2	0.89 2			0.89 2		0.89 2		
Pierced	130	46.92	61	0.76 1	0.76 1			0.76 1				
Uninjected	631	10.93	69	0.79 5	0.47 3	0.31 2		0.15 1		0.31 2		0.15 1

SUMMARY - ALL DOSE LEVELS

598	57.19	342	1.00 6	1.00 6	0.17 1	0.17 1	0.33 2	0.33 2	0.17 1	0.33 2	
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TABLE 4
K Nitrite

in Water

Yolk - 96 hrs

Dose, ppm	No. Fertile	Mortality % #		Abnormal Total % #		H-S-V-L % #		Abnormalities by category										
								Head % #		Skeletal % #		Viscera % #		Limbs % #		Struc- tural % #		Toxic Response % #
1000.	110	93.63	103	0.00	0	0.00	0											
500.	110	100.00	110	0.90	1	0.90	1	0.90	1									
300.	162	24.69	40	3.08	5	0.61	1	0.61	1						2.46	4		
200.	162	17.28	28	3.70	6	3.70	6	0.61	1		0.61	1	2.46	4		0.61	1	
100.	161	9.93	16	0.00	0	0.00	0											
20.0	52	11.53	6	1.92	1	1.92	1	1.92	1									
10.0	52	9.61	5	1.92	1	0.00	0								1.92	1		
0.0	211	15.16	32	0.47	1	0.47	1	0.47	1									
Pierced	158	15.82	25	1.89	3	1.89	3	1.26	2				0.63	1				
Untreated	631	10.93	69	0.79	5	0.47	3	0.31	2		0.15	1		0.31	2		0.15	1

SUMMARY - ALL DOSE LEVELS

809	38.07	308	1.73	14	1.11	9	0.49	4		0.12	1	0.49	4		0.74	6
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Table 5
K Nitrite
In Water
Chi-Square Analyses of Mortality

Dose Level mg/kg	Air Cell		Yolk	
	0 hrs	96 hrs	0 hrs	96 hrs
9.96	—	—	4.91*(less)	—
10.00	—	2.39	5.31*(less)	0.65
19.80	—	—	4.91*(less)	—
20.00	—	20.25*	—	0.20
60.00	—	73.53*	—	—
80.00	—	121.91*	—	—
99.60	—	—	0.10	—
100.00	1.26	97.22*	0.41	1.78
199.80	—	—	0.25	—
200.00	2.91	210.25*	6.58*	0.17
300.00	49.57*	241.62*	10.56*	4.74*
500.00	139.76*	208.36*	66.26*	207.53*
1000.00	137.25*	96.57*	—	179.50*
All Doses (DF)	326.99*(5)	575.85*(9)	136.38*(9)	519.50*(7)

* Probability $< 0.05 - 0.005$.

Table 6
K Nitrite
In Water
Probit Analyses - Mortality

	Air Cell			Yolk	
	0 hrs	96 hrs		0 hrs	96 hrs
LD-30 (Range)	230.0 (44.9-305.1)	25.6 (11.3-40.2)	NS	313.7 (166.5-404.6)	
LD-50 (Range)	278.9 (134.9-422.3)	47.1 (27.1-67.7)	NS	387.4 (273.1-547.2)	
LD-70 (Range)	338.2 (244.4-968.3)	86.9 (59.4-124.1)	NS	478.3 (370.8-894.7)	
LD-90 (Range)	446.7 (331.5-5581.6)	210.1 (144.5-379.9)	NS	648.5 (483.3-2170.1)	

Table 7
K Nitrite
in Water
Chi-Square Analyses of Abnormalities

Dose Level mg/kg	Air Cell		Yolk	
	0 hrs	96 hrs	0 hrs	96 hrs
9.96	—	—	0.76	—
10.00	—	0.18	0.29	0.04
19.80	—	—	0.29	—
20.00	—	0.31	—	0.04
60.00	—	0.11	—	—
80.00	—	0.26	—	—
99.60	—	—	0.76	—
100.00	0.01	1.95	0.03	0.02
199.80	—	—	0.76	—
200.00	3.01	0.67	0.03	3.59
300.00	0.74	1.93	1.51	2.47
500.00	0.74	1.09	0.03	0.08
1000.00	0.00	0.00	—	0.11
All Doses (DF)	8.33 (5)	20.16*(9) (less)	13.95 (9)	14.37*(7)

* Probability < 0.05-0.005.

TABLE 8
K Nitrite
in Water
Probit Analyses - Abnormalities

Air Cell		Yolk	
0 hrs	96 hrs	0 hrs	96 hrs
NS	NS	NS	NS

Table 9
K Nitrite
In Water
Chi-Square Analyses of HLSV Abnormalities

Dose Level mg/kg	Air Cell		Yolk	
	0 hrs	96 hrs	0 hrs	96 hrs
9.96	—	—	0.76	—
10.00	—	0.18	0.03	0.58
19.80	—	—	0.29	—
20.00	—	1.47	—	0.04
60.00	—	0.11	—	—
80.00	—	0.17	—	—
99.60	—	—	0.76	—
100.00	0.01	1.95	0.03	0.02
199.80	—	—	0.76	—
200.00	0.00	0.27	0.03	2.47
300.00	0.74	1.93	1.51	0.28
500.00	0.01	1.09	0.03	0.08
1000.00	0.00	0.00	—	—
All Doses (DF)	5.75 (5)	21.66* (9) (less)	16.90 (9)	10.68 (6)

* Probability < 0.05-0.005.

TERATOGENIC FINDINGS

TREATMENT	TOTAL NO. EXAMINED	TOTAL NO. ABNORMAL	SPECIFIC FINDINGS													
			NO.	D	E	S	C	R	I	P	T	I	O	N		
Untreated Control	631	5	2	dysgnathia -beak												
			1	agenesis-down; celosomia-abdomen												
			1	ataxia												
			1	dwarfism												
Drilled Control - 0 hrs	230	3	2	abnormal curvature - femur, bilateral												
			1	abnormal shortening - maxilla and beak												
Drilled Control - 96 hrs	373	0	0													
Pierced Control - 0 hrs	130	1	1	celosomia - abdomen												
Pierced Control - 96 hrs	158	3	1	exencephaly; aplasia - down												
			1	abnormal curvature - hindlimb, unilateral												
			1	exencephaly												
Air Cell - 0 hrs 1000.0 mg/kg	109	0	0													
500.0	116	2	1	dipygus												
			1	triocephalus												
300.0	116	2	1	anophthalmia - bilateral												
			1	fusion failure - abdomen												
200.0	152	5	1	vacuolization-liver; graunlation tissue-renal tubule												
			4	vacuolization-liver												

TERATOGENIC FINDINGS

TREATMENT		TOTAL NO. EXAMINED	TOTAL NO. ABNORMAL	TERATOGENIC FINDINGS	
				NO.	SPECIFIC FINDINGS
Air Cell - 0 hrs	100.0 mg/kg	117	1	1	anophthalmia-unilateral; dysgnathia-beak
	0.0	143	0	0	
Air Cell - 96 hrs	1000.0	28	0	0	
	500.0	110	0	0	
	300.0	162	0	0	
	200.0	162	7	1	agenesis-head and hindlimb
				1	hemorrhage-head
				1	agenesis-head
				4	abnormal curvature-toe
	100.0	163	0	0	
	80.0	48	1	1	agenesis-kidney; hypopigmentation-down
	60.0	43	0	0	
	20.0	134	1	1	malposition
	10.0	52	1	1	abnormal curvature-toe
	0.0	182	4	1	anophthalmia-unilateral; dysgnathia-beak
				1	agenesis-wing, unilateral and hindlimb, unilateral
				1	microphthalmia-bilateral; dicephalus (2 heads)

TABLE 10
K Nitrite in Water

Sheet 3

TERATOGENIC FINDINGS

TERATOGENIC FINDINGS														
TREATMENT		TOTAL NO. EXAMINED	TOTAL NO. ABNORMAL	SPECIFIC FINDINGS										
				NO.	D	E	S	C	R	I	P	T	I	O
Yolk - 0 hrs	500.0 mg/kg	100	0	0										
	300.0	119	4	1	abnormal curvature-toe, unilateral									
				1	displacement-sternum; umbilical cord around fetus									
				1	celosomia-abdomen									
				1	micromelia-wing, bilateral; phocomelia-hindlimb, bilateral; agenesis-down									
	200.0	99	0	0	0									
	199.8	20	0	0										
	100.0	100	0	0										
	99.6	20	0	0										
	19.8	20	1	1	exencephaly; celosomia-abdomen									
	10.0	100	1	1	hemorrhage-umbilical cord									
	9.96	20	0	0										
	0.0	224	2	2	dwarfism; celosomia-abdomen									
Yolk - 96 hrs	1000.0	110	0	0										
	500.0	110	1	1	dysgnathia-beak									
	300.0	162	5	4	edema-hindlimb, bilateral									
				1	exencephaly; dysgnathia-beak									

TABLE 10
K Nitrite in Water

Sheet 4

TERATOGENIC FINDINGS

[illegible]

TABLE 11

POST-HATCH DATA

POTASSIUM NITRITE

Injection Date - 11/15/71

TREATMENT		Age at Sexual Maturity (days)	B O D Y W E I G H T				Feed Consumption		
Label	Dose Level (mg/kg)		At Hatch (gm)	6 wks. gm		6 mo. kg		6 wks gm	6 mos kg
				M	F	M	F		
204	200	145	43.1	397	334	1.85	1.66	737	10.9
205	100	150	40.7	406	371	1.73	1.72	857	10.9
206	H ₂ O	144	41.9	440	384	2.09	1.56	899	10.5
208	Untreated	148	42.5	436	383	1.53	1.56	855	10.5